DALHOUSIE

2016 Protocols

Illumina MiSeq 16S rRNA Sequencing

Adapted from:
A custom and streamlined workflow for microbiome research
A. M. Comeau, G. M. Douglas and M. G. I. Langille (manuscript in progress)
.

Purpose:
To sequence 16S microbial DNA isolated from animal fecal matter and perform compositional analysis of bacterial communities.

Reference:
This protocol was adapted from:
A custom and streamlined workflow for microbiome research
A. M. Comeau, G. M. Douglas and M. G. I. Langille (manuscript in progress)

Required Materials:

- Single-channel pipettes (2-1000 µL)
- Multi-channel (8) pipette(s) (MCP; 2-100 µL)
- Required equipment for the DNA extraction kit(s) of your choice
- PCR machine with 96-well block
- *Invitrogen Mother E-Base* (#EB-M03) for *E-Gels 96*
- Documentation system for DNA gels (SYBR Safe filter)
- Microvolume DNA fluorescence reader (such as *Qubit w/PicoGreen*)
- *Illumina Experiment Manager (iEM)* software
- *Illumina MiSeq* sequencer with *RTA v1.17.28 / MCS v2.2* or later
- Bead-mill / Tissue lyser / Homogenizer (if using bead-based DNA extractions)
- Microvolume DNA spectrophotometer (such as *NanoDrop*; for quantifying extracts)
- Centrifuge with rotor for 96-well plates (useful for spinning down condensation)
- Standard gel electrophoresis system (for analyzing recalcitrant samples)
- Pipette filter tips (p2 to p1000)
- Reagent reservoirs for MCP
- 1.5 mL Eppendorf tubes
- DNA extraction kit(s)
- 96-well thin-wall 0.2 mL PCR plates (such as *Bio-Rad* #HSP9601)
- PCR plate films (such as *Bio-Rad* #MSB1001)
- *Thermo Phusion High-Fidelity DNA Polymerase* (#F-530L) or similar
- dNTP mix (at 40 mM = 10 mM of each base)
- *Illumina* fusion primers (see below and Excel template)
- PCR-grade water
- *Invitrogen E-Gels 96 2% with SYBR Safe* (#G7208-02)
- *Invitrogen E-Gel Low Range Ladder* (#12373-031; diluted 1:1 with PCR-grade water)
- *Invitrogen SequalPrep Normalization Kit* (#A10510-01)

- *Qubit/PicoGreen* assay tubes (clear 0.2 mL)
- 2 N NaOH
- 200 mM Tris-HCl, pH 7
- *Illumina PhiX Control Kit v3* (#FC-110-3001)
- *Illumina MiSeq Reagent Kit v3 (600 cycle)* (#MS-102-3003)
- Ethanol (usually a requirement for extraction/purification kits)
- Thin-wall 0.2 mL PCR tubes or strips with caps (for re-amplifying recalcitrant samples)
- Agarose, loading buffer/stain and 100 bp ladder (for analyzing recalcitrant samples)
- PCR product purification kit (if need to concentrate final library)

Procedure:

1. Copy existing 16S/18S primers or to design your own custom gene primers with the proper Illumina indices and Nextera adaptor orientations. We order IDT "Ultramers" (www.idtdna.com) for such long primers (~80-90 nt) as their coupling efficiency is one of the highest available (critical for obtaining high proportions of full-length oligos in the mix you obtain). Order the fusion primers at 4 nmole scale in deep-well plates; one set per 96-well plate.
2. Once arrived, add 400 µL of PCR-grade water to each well containing the primers in order to reconstitute them at a concentration of 10 µM (1/10th the typical 100 µM working stock concentration for primers).
3. Prepare the 1 µM working stock **Forward Set 1 Primer Plate** by pipetting 63 µL of PCR-grade water into each well of the 96-well PCR plate from a sterile reservoir. Rotate the deep-well primer plate 90° clockwise and align it so that the 8 occupied wells (= 8 different indices) of row 1 line up with the 8 rows of the new plate. Once complete, each column of the resulting plate will have enough primer for one complete 96-well plate PCR (leaving some extra room for pipetting error; 12 columns X 5 µL = 60 µL required). Seal the plate with PCR film and store at -20°C.
4. Prepare the 1 µM working stock **Forward Set 2 Primer Plate** by repeating step **3**, but using row 3 of the reconstituted deep-well primer plate.
5. Prepare the 1 µM working stock **Reverse Set 1 Primer Plate** by pipetting 45 µL of PCR-grade water into each well of the 96-well PCR plate from a sterile reservoir. Align the deep-well primer plate horizontally (normal orientation) so that the 12 occupied wells (= 12 different indices) of row 5 line up with the 12 columns of the new plate. Once complete, each row of the resulting plate will have enough primer for one complete 96-well plate PCR (leaving some extra; 8 rows x 5 µL = 40 µL required). Seal the plate with PCR film and store at -20°C.
6. Prepare the 1 µM working stock **Reverse Set 2 Primer Plate** by repeating step **v**, but using row 7 of the reconstituted deep-well primer plate.
7. Once all aliquoting is complete, seal the deep-well plate with PCR film and archive

at -20°C until new aliquots are required (minimized freeze-thaw cycles).

8. Extract DNAs from your samples using the method/kit appropriate to the specific samples (ex: generally stool [with bead-beating kits], but also urine, etc.).
9. Quantify and quality-check your final DNAs via *NanoDrop* or *Qubit/PicoGreen* to verify success.
10. Aliquot 5 µL of each of the 380 DNA samples into 4 × 96-well PCR plates (**DNA Plates 1-4**) in the order desired (we prefer by column), allowing for one PCR negative control well (position H12) on each plate.
11. Prepare the following PCR master-mix for **Plate 1** in a 1.5 mL Eppendorf tube:

	Per 25 µL reaction	**X 100 reactions**
5 x HF PCR Buffer	5 µL	500 µL
dNTPs (40 mM)	0.5 µL	50 µL
F primer (1 µM)	5 µL	Added to each, after
R primer (1 µM)	5 µL	Added to each, after
***Phusion* (2 U/µL)**	0.25 µL	25 µL
PCR-grade Water	7.25 µL	725 µL
Template	2 µL	Added to each, after

12. Dispense 78.5 µL of the master-mix into the 16 wells of 2 columns (or 105 µL in 12 wells of one row) of a 96-well plate (remaining wells to be used in subsequent PCR preps) – this plate now becomes the **Master-Mix Plate** and is used to transfer the master-mix into the PCR plate using an MCP.
13. Dispense 13 µL of master-mix into each well of the **PCR Plate 1 (2 µL)** one column (or row) at a time with the MCP.
14. Remove the protective film ("uncover") from one column of the **Forward Set 1 Primer plate** align it horizontally on the bench to the left of **PCR Plate 1 (2 µL)** and dispense 5 µL into each well, one column at a time using the MCP.
15. Uncover one row of the **Reverse Set 1 Primer Plate**, align it vertically on the bench along the top of **PCR Plate 1 (2 µL)** and dispense 5 µL into each well, one row at a time using the MCP.
16. Uncover the **DNA Plate 1**, align it along the top of **PCR Plate 1 (2 µL)** and dispense 2 µL into each well, one column at a time using the MCP.
17. Once complete, seal the plate with PCR film, place in a thermocycler and run the following program:

	Temperature	**Time**
Initial Denaturation	98°C	30 s
Denaturation	98°C	10 s
Annealing	55°C	30 s
Extension	72°C	30 s
Final Extention	72°C	4:30
Hold	4°C	∞

18. While the first PCR is running, prepare the 1/10th dilution of **DNA Plate 1** – add

27 µL of PCR-grade water to each well of the remaining 3 µL of template in **DNA Plate 1**, for a total of 30 µL final volume per well, using a reservoir (~3 mL required) and MCP.

19. Once the first PCR is complete (or nearly so), repeat steps **11-17** to prepare **PCR Plate 1 (0.2 µL)** using the newly diluted templates.
20. Once the two PCRs for **Plate 1** are complete, repeat steps **11-19** to prepare **PCR Plates 2 (2 µL) & (0.2 µL)** from **DNA Plate 2** using **Forward Set 1 Primer Plate** and **Reverse Set 2 Primer Plate.**
21. Once the two PCRs for **Plate 2** are complete, repeat steps **11-19** to prepare **PCR Plates 3 (2 µL) & (0.2 µL)** from **DNA Plate 3** using **Forward Set 2 Primer Plate** and **Reverse Set 1 Primer Plate.**
22. Once the two PCRs for **Plate 3** are complete, repeat steps **11-19** to prepare **PCR Plates 4 (2 µL) & (0.2 µL)** from **DNA Plate 4** using **Forward Set 2 Primer Plate** and **Reverse Set 2 Primer Plate.**
23. Plug in the *Mother E-Base*, unwrap a fresh *E-Gel 96* and insert it into the base.
24. The duplicate PCR reactions of **Plate 1** are aggregated then loaded onto the gel in the same action: using the MCP and working by rows (the gel cannot be loaded by columns as they are staggered), pipet 20 µL out of the **PCR Plate 1 (0.2 µL)** into the corresponding wells of **PCR Plate 1 (2 µL)** and mix by pipetting up and down, then take 20 µL of this aggregate and load into the appropriate wells of the gel.
25. Once all rows are complete, load 20 µL of the *E-Gel Low Range Ladder* into some of the marker ("M") wells, then run the gel for the pre-set 12 min.
26. Visualize the gel and photograph on a UV/blue transilluminator with a SYBR filter.
27. Repeat steps **23-26** for **PCR Plates 2 (2 µL) & (0.2 µL)**.
28. Repeat steps **23-26** for **PCR Plates 3 (2 µL) & (0.2 µL)**.
29. Repeat steps **23-26** for **PCR Plates 4 (2 µL) & (0.2 µL)**.
30. Any samples with failed PCRs (or spurious bands) are re-amplified by optimizing the PCR (further template dilution to 1:100 or using BSA/other additives) to produce correct bands in order to complete the amplicon plate. Unless this represents the majority of a plate (in which case continue with plates and E-gels), PCRs are done in standard tubes/strips and visualized using a traditional gel box. Once correct bands have been obtained, amalgamate those few tubes into the appropriate wells of the respective **Aggregated PCR Plates** before continuing.
31. Use the remaining 20 µL of each well in the **Aggregated PCR Plate 1** to cleaned-up and normalize the amplicons using the high-throughput *Invitrogen SequalPrep 96-well Plate Kit*. Label this final plate **SequalPrep Plate 1**.
32. Once the *SequalPrep* protocol is complete, pool the 95 samples from **SequalPrep Plate 1** by using the MCP to transfer 5 µL of each column into one column of a new 96-well plate named the **Library Pool Plate** (remaining columns to be used in subsequent pooling). Once complete, pipette 50 µL of each of the 8 wells into one 1.5 mL Eppendorf tube and label **Plate 1 Library Pool**.
33. Repeat steps **31 & 32** for **Aggregated PCR Plate 2**.
34. Repeat steps **31 & 32** for **Aggregated PCR Plate 3**.
35. Repeat steps **31 & 32** for **Aggregated PCR Plate 3**.
36. Once all four pools are complete, pipette 100 µL of each of the four tubes into

one 1.5 mL Eppendorf tube and label **Final Library Pool** (add the run name to the tube or some other identifier to keep your various pools separate).

37. Quantify the **Final Library Pool** using the *Invitrogen Qubit with PicoGreen* (or similar fluorescence-based alternative; 5 µL of pool to be assayed) and calculate the molar concentration using the following formula, knowing that 1 ng/µL of a 500 bp amplicon = 3.29 nM:

    (500 *bp / size in bp of amplicon*) x (*concentration in ng/µL*) x (3.29)

38. This section is based upon the following *Illumina* documents, with some small procedural changes (including using the *NextSeq* variant for sample denaturation), and the inclusion of instructions to be able to load >96 samples (i.e.: 384 combinations of indices) which are not written out by *Illumina* – familiarize yourself with these documents / have them on-hand:
    - *MiSeq Reagent Kit v3 – Reagent Preparation Guide*
    - *Preparing Libraries for Sequencing on the MiSeq*
    - *Denaturing and Diluting Libraries for the NextSeq 500*
    - *MiSeq System User Guide*

39. Begin thawing the *v3 Reagent Cartridge* and tube of *HT1* as instructed. Put at 4°C when complete.
40. While waiting, prepare the *Sample Plate* and *Sample Sheet* files that will be used to run the *MiSeq* by opening the *Illumina Experiment Manager (iEM)* software.
41. Create the *Sample Plates* first – in order to run all 384 combinations of indices, 4 separate *Sample Plates* (one per plate from our protocol above) will be required.
42. Now create the 4 *Sample Sheets* that correspond to the *Sample Plates*, starting with the first plate.
43. Now merge the 4 *Sample Sheet* files, which are in CSV format, into the one final file which will run the *MiSeq*.
44. Prepare 0.2 N NaOH as instructed, except make 10-fold less (20 µL of 1 N NaOH + 80 µL of water).
45. Use the nanomolar concentration from step **37** to determine whether the final amplicon library must be diluted or concentrated prior to continuing. A fixed concentration of 4 nM is the standard requirement for the *MiSeq*, however, using the *NextSeq* loading protocol, a library between 0.4-4 nM can be accommodated by simply using a larger volume of a more dilute library.
46. Denature the library, using the appropriate volume of 0.2 N NaOH, for 5 min. at room temperature.
47. Neutralize the reaction by adding the equivalent volume of Tris-HCl
48. Dilute out the library to 20 pM using the appropriate amount of chilled *HT1* and place on ice.
49. Combine 570 µL of the library with 30 µL (= 5%) of the already diluted and denatured *PhiX Control Library*.

50. Proceed with loading the 600 µL sample in the *v3 Reagent Cartridge* and continue the *MiSeq* run start procedure.